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Structural Difference Between Walls from Hemispherical Caps and Partial Septa of *Bacillus subtilis*

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Walls from partial septa of *Bacillus subtilis* bacteria are more sensitive than end walls to digestion by *B. subtilis* autolytic amidase. This result indicates that, after synthesis, *B. subtilis* septal walls are modified to an amidase-resistant form.

Recent experiments (3) have shown that walls from the cylindrical zone (sides) of *Bacillus subtilis* cells are structurally different from those at the hemispherical caps (ends). In those experiments, isolated *B. subtilis* walls were digested with *B. subtilis* autolysin in the presence of 0.3 M LiCl (Fig. 1). The side walls were preferentially degraded so that there was a period during the digestion when the side walls were completely solubilized and the only morphologically recognizable wall structures remaining were disks and rings. The disks obviously came from the end walls which were relatively resistant to digestion. However, the source of the rings was not clear.

Based on the data of Fig. 1, it was possible to consider basically two models for end wall and septal wall synthesis (Fig. 2, models I and III). in model I, the end wall is made in autolysin-sensitive form. However, there is a subsequent modification of the end wall so that it becomes autolysin resistant. The modification proceeds from the periphery of the end and approaches the center. Therefore, if an end wall is isolated before the modification has been completed, there will be a small area at the center of the end which is unchanged and hence sensitive to autolysin. Digestion of this end wall will leave a ring of resistant material such as seen in Fig. 1 (right frame). In this model both walls from partial septa (partial septal walls) and new end walls are unmodified and sensitive to degradation. At the other extreme is model III (Fig. 2) where the partial septal walls, which eventually give rise to end walls, are already resistant to autolysin. In this model, the rings seen after digestion (Fig. 1, right frame) would have come from partial septa. For the sake of completeness, model II (Fig. 2) is also considered. In this model, modification of the sort described for model I begins before septal wall is completed, in which case the rings (Fig. 1, right frame) may have arisen from either incompletely modified partial septal or end walls.

One way of testing the models in Fig. 2 is to determine unambiguously the resistance of partial septal walls to digestions. If no partial septal walls are resistant to autolysin, model I is correct. Conversely, if all the rings (Fig. 1, right frame) come from partial septal walls, with the hole in the center being the same size before and after digestion, then model III is valid. If partial septal walls give a resistant ring after digestion, with the diameter of the hole being larger after digestion than before, model II is the one of choice.

In the experiments in this paper it was possible to follow the digestion of individual partial septal walls. The results clearly show that model I is usually the correct one.

MATERIALS AND METHODS

Preparation of B. subtilis walls and autolysins for digestion on grids. Walls were isolated from exponential-phase *B. subtilis* βAO bacteria growing in broth culture at 37 C and then were treated with sodium dodecyl sulfate (SDS) to destroy all autolysins attached to the walls (1). The autolysin preparation, (3 M LiCl autolysin) was freshly isolated *B. subtilis* βAO walls extracted with 3 M LiCl in TK buffer (0.1 M KCl, 0.1 M tris[hydroxymethyl]aminomethane, pH adjusted to 8.6 with HCl) (1).

Electron microscopy for digestion on grids. Since our experiments involved the photography of a sample before and after enzyme digestion, H-2 London 200

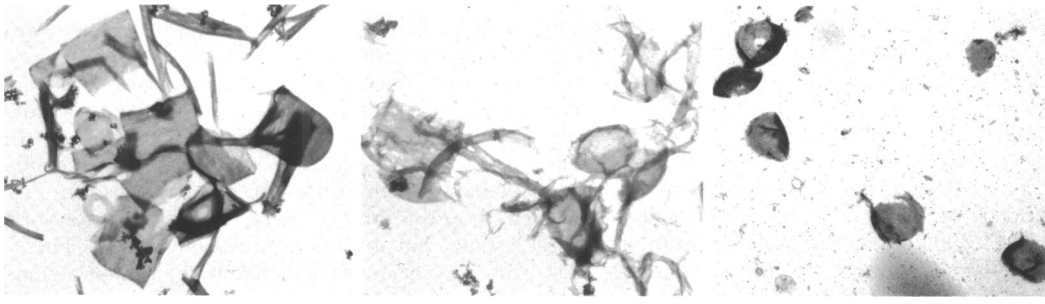

FIG. 1. *Morphology of B. subtilis walls at various stages of autolysis. The procedure was as previously described (3). Native B. subtilis βAO bacterial walls were allowed to autolyse in 0.3 M LiCl at pH 8.6 at 45 C. Samples were withdrawn at various times, attached to carbon-coated electron microscope grids, stained with uranyl acetate, and photographed in the electron microscope. Left frame, Beginning of the autolysis incubation. Center frame, After the absorbancy at 540 nm decreased to 35% of the predigestion value. Note that the side walls had been partially digested. Right frame, After the absorbancy at 540 nm had decreased to 13% of the predigestion value. Note that the side walls had been completely digested. At this time most of the objects seen were disks, but a few rings were also present as shown in the upper left-hand corner of this frame. ×6,000.*

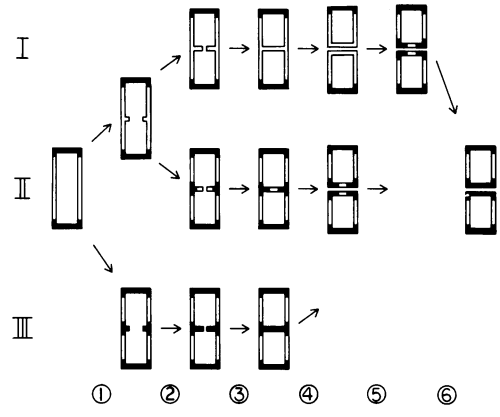

FIG. 2. *Models for B. subtilis end wall growth. Walls of the bacteria are pictured in median cross-section along the axis of the cells. Autolysin-sensitive regions are white, and autolysin-resistant regions are black. The resistant end walls are drawn to overlap slightly the side wall regions because thin-section electron micrographs (2, 4) of B. subtilis bacteria show that the regions morphologically identifiable as end walls indeed do extend a small distance into the cylindrical portions of the cells. For all models: step 1 is the beginning of septal wall formation; step 2 is further synthesis of septal wall; step 3 is completion of septal wall; step 4 is cell separation. Model I: Septal wall material is synthesized in autolysin-sensitive form. After cell separation, the end walls are modified to a resistant form. The modification begins at the periphery of the ends (step 5) and progresses toward the center (step 6). Model II: Same as model I except that the modification begins after septal wall synthesis has begun and before septal wall synthesis has finished. Model III: All septal wall material is synthesized in autolysin resistant form from the beginning of septal wall synthesis.*

finder grids (from Graticules, Ltd., London, England through Ernest F. Fullam, Inc., Schnectady, N.Y.) were used to facilitate the process of finding the sample after digestion. Formvar-coated H-2 grids were surface ionized in a glow discharge apparatus in a vacuum evaporator. A small drop of bovine serum albumin (0.1% in water) was placed on the grid and removed with a piece of filter paper. A suspension of *B. subtilis* SDS walls in water was applied to the grid and removed after 1 min. A small drop of water saturated with uranyl acetate was applied and removed after 0.5 min. The grid was then washed with 1 drop of water, and negatives were taken in a Hitachi HU-11C electron microscope fitted with a decontamination device using DuPont Cronar COS-7 film at a magnification of ×2,400. The grid was removed from the microscope and floated on a drop of solution containing 4 to 6 μliters of 3 M LiCl autolysin and 45 μliters of TK buffer for 20 min at 45 C. The enzyme and buffer solutions were mixed on a plastic petri dish by stirring with a small plastic rod before application of the grid. During the incubation the petri dish was covered with its lid and placed just above the water level in a covered water bath at 45 C. At the end of the incubation the grid was touched to a filter paper to remove excess liquid; the grid was then stained, washed, and photographed as before digestion.

RESULTS

The main goal of our experiments was to study the degradation of partial septal walls by *B. subtilis* autolysins under conditions where end walls were degraded more slowly than side walls. Since the pattern of degradation of partial septal walls could not be inferred unambiguously by comparing electron micrographs of walls at various stages of degradation, we developed another method for studying wall digestion. In this technique, isolated walls were

absorbed to a Formvar film on an electron microscope grid, stained with uranyl acetate, and photographed in the electron microscope. The grid was removed from the microscope and floated on a drop of *B. subtilis* autolysins which digested the stained walls. The enzyme preparation used contained all the autolysins that could be removed from *B. subtilis* walls with 3 M LiCl. Therefore, the majority autolysin was the *N*-acyl-muramyl-L-alanine amidase (alanine amidase), but traces of glycosidase were also present (1). The digestion was performed in approximately 0.3 M LiCl at pH 8.6 at 45 C because it had been found previously that, under these conditions, the end walls were more stable to degradation than side walls. The digestion was entirely due to the amidase because the glycosidase is inactive in 0.3 M LiCl at pH 8.6. After digestion, the grid was restained with uranyl acetate and photographed a second time. By comparing the micrographs from before and after digestion it was possible to trace unambiguously the digestion of particular walls (Fig. 3, 4, and 5). All the walls obviously stayed on the grid without changing position during the manipulations between photographs. The orientations of walls relative to one another were never altered, but sometimes the distances between walls would change a very small, but noticable, amount between photographs. This could be due to slight stretching or shrinking of the Formvar film.

Furthermore, the entire surface of the wall was adsorbed to the Formvar film. This fact can be seen by examining partially degraded side walls (Fig. 3, 4, and 5). Many of the fragments remaining after partial digestion were no longer in contact with one another. Nevertheless, the outline of the wall before digestion could be seen. Thus all the fragments must have been attached independently to the film and in a sufficient number of sites to keep their orientations. When the grid was incubated with buffer to replace the autolysin preparation, the micrographs taken before and after incubation were identical.

After partial digestion, the side walls began to have holes and sometimes the holes were not round but were elongated with the long axis parallel to the end of the cell (Fig. 3, 4, and 5). Also, it is clear that usually the end walls were more resistant to degradation than the side walls (samples K, Fig. 3, 4, and 5). These same morphologies were seen when freshly isolated walls were allowed to autolyse in 0.3 M LiCl (Fig. 1). Therefore, the wall digestion pattern was very little affected by staining with uranyl acetate before degradation on grids. There was some non-uniformity of digestion over the surface of any given grid so that in some regions the walls after digestion became uniformly indistinct and no detail could be distinguished. In other areas the texture of partially digested side walls was quite different from that seen in Fig. 3, 4, and 5, and in these regions the ends were usually not more resistant to digestion. Therefore, attention was only focused on those regions of the grid where the digestion pattern for side walls was like that seen during autolysis of freshly prepared walls in 0.3 M LiCl (Fig. 1). In those regions, 82% (Table 1) of the structures unambiguously identifiable as end walls were resistant to digestion. Therefore, it seems reasonable to conclude that most end walls were resistant to digestion under the appropriate conditions.

The quality of the micrographs taken before digestion was sometimes less than optimal because it was necessary to minimize the electron beam exposure to the photographed regions. Therefore, the grid was first focused in a region close to the one photographed. The exposure conditions were adjusted to the minimum which still permitted the taking of negatives of sufficient contrast to identify partial septa, and then a previously unexamined region was moved under the beam and left just long enough to expose the film.

Excess exposure had two deleterious effects. When a region was left under the electron beam for a time sufficient to permit focusing, the digestion pattern was very atypical, with the ends no longer being preferentially resistant. In addition, the walls tended not to stay attached to the Formvar film, and very often no walls would remain after the digestion incubation. The change in digestion pattern with over-exposure may have resulted from electron damage to the sample, whereas the detachment of walls might have been a consequence of electron beam induced charge changes in either the film or the walls so that they repelled each other. If the beam exposure were continued even longer, the walls became attached to the grids by contamination which also prevented any subsequent digestion. Fortunately, the electron beam only adversely affected the region directly exposed so the focus and exposure adjustments could be performed at leisure in an area near the one photographed.

In negatives taken before digestion, partial septal walls were seen in either of two views. In the end view, there was a regularly shaped ring of uniform width (Fig. 3; samples A, B, and C).

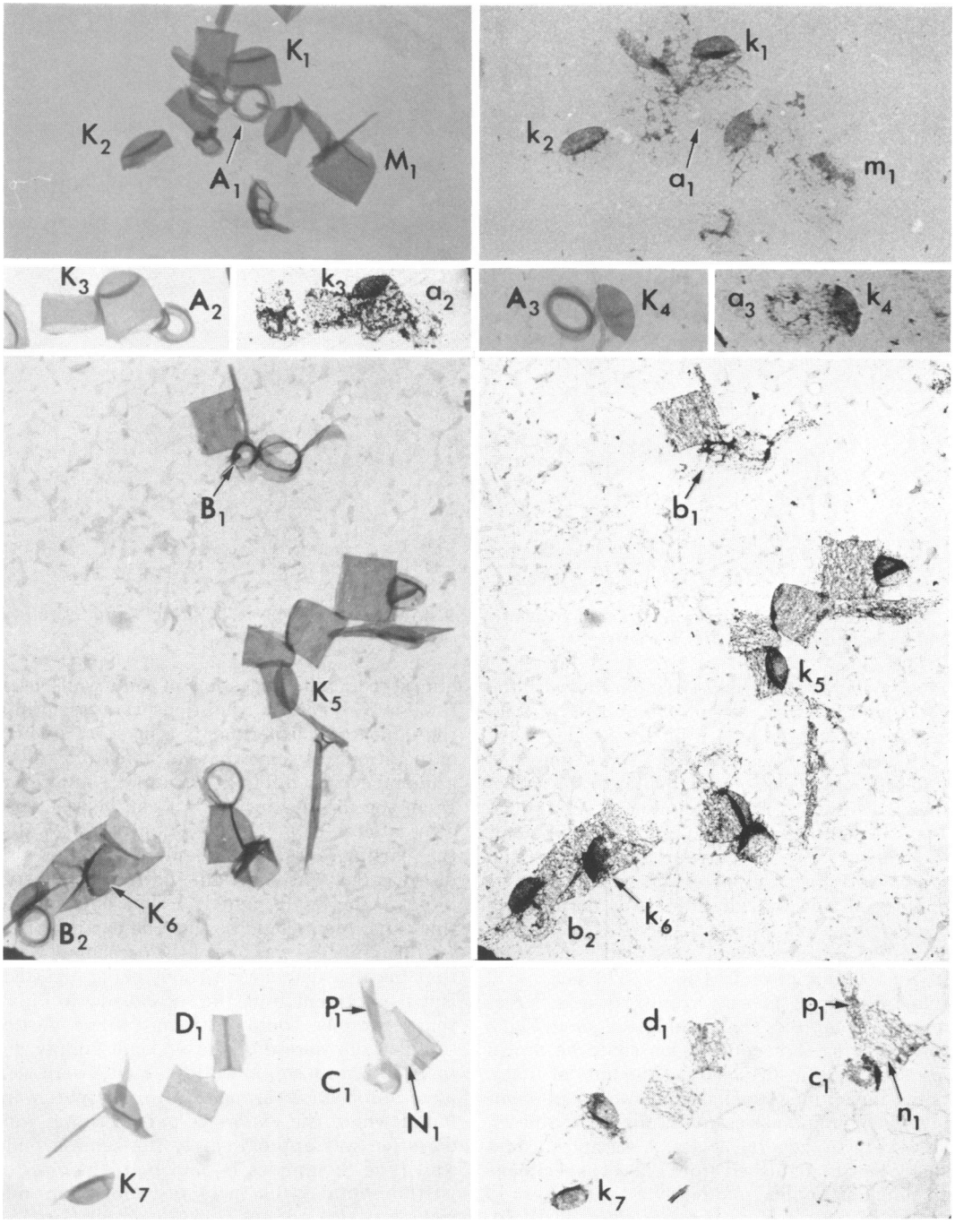

Fig. 3. *B. subtilis autolysin digestion of B. subtilis walls—primarily end views of partial septa. B. subtilis walls were digested on electron microscope grids as described in Materials and Methods. The left-hand frame of each pair of micrographs shows the walls before digestion and the right-hand frame shows the walls after digestion. The letters indicate the various types of wall structures (Table 1). The numbers index particular pairs of interesting structures.* ×7,200.

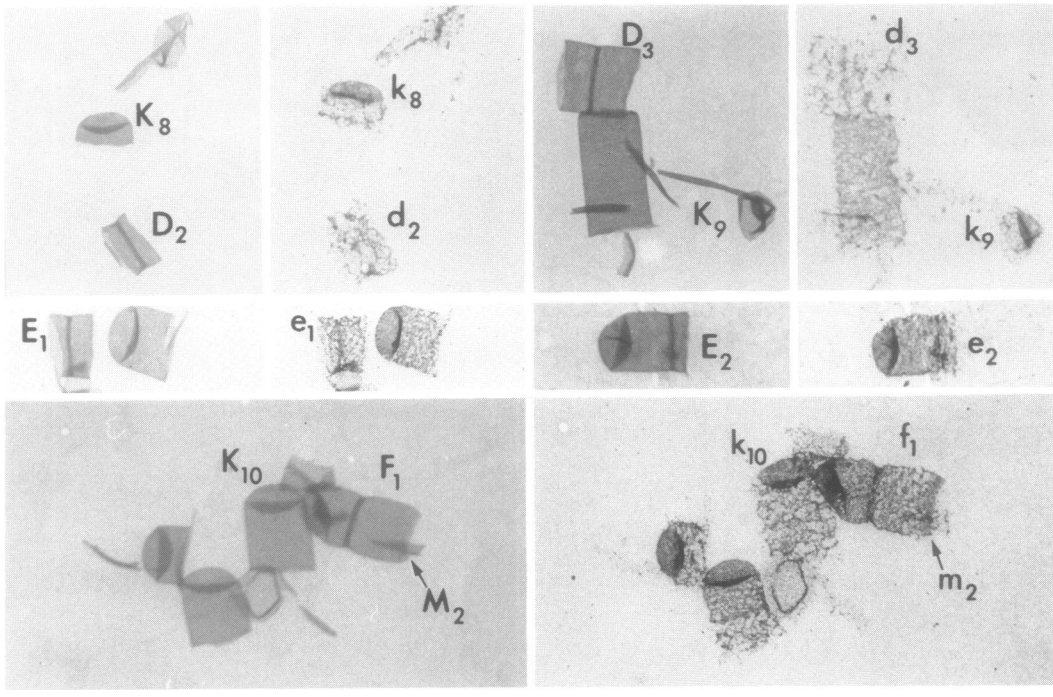

Fig. 4. *B. subtilis autolysin digestion of B. subtilis walls—primarily side views of partial septa. Notations and procedures are as for Fig. 3.* ×7,200.

These partial septal walls lying parallel with the Formvar film were often surrounded by fragments of collapsed side wall. In the side view, there was typically a heavy bar in a segment of side wall which was perpendicular to the axis of the wall (Fig. 3, 4, and 5; samples D, E, and F). The examples chosen for Fig. 3, 4, and 5 show the entire range of patterns seen for partial septal wall digestion in regions where the morphology of side wall digestion was normal and where end walls were protected. The various digestion patterns were classified, and their frequencies are given in Table 1. The purpose of this table is to present results from a larger number of samples than could be given in Fig. 3 through 5 so that conclusions could be drawn from a significantly large amount of data. Sometimes the classifications were not completely rigorous (classes A through F, Table 1). However, in general, class A samples could easily be distinguished from class C specimens although examples from either class often could not be separated from those of class B. Analogous observations hold for classes D through F.

Figure 3 shows primarily end views of digestion of partial septal walls. From comparisons of the various pairs of micrographs, it is clear that the extent of digestion ranged from partial to almost complete digestion of side walls even though the digestion conditions were essentially the same for all experiments. The exact amount of digestion was somewhat variable from grid to grid and even in different areas of the same grid. From the top row in Fig. 3 it can be seen that after almost complete digestion of side walls, the partial septal wall (sample A_1) had also disappeared. The end walls, however, were still intact (samples K_1 and K_2). The objection to this experiment could be that the partial septal wall (sample A_1) was one of the occasional walls that became detached without being digested. But it is evident from the second row in Fig. 3 that examples could be found where partial septal walls were digested and not simply detached because septal wall fragments were seen after autolysin treatment. Also, samples were found where the extent of partial septal wall digestion was approximately the same as side wall (Fig. 3, samples B_1 and B_2). The class of partial septal walls more resistant than side walls was also present (Fig. 3, sample C_1). The relative frequencies of the different types of digestion are given in Table 1. These figures show that the majority of the partial septal walls, unlike end walls, are at least as degradable as side walls. In the two examples found

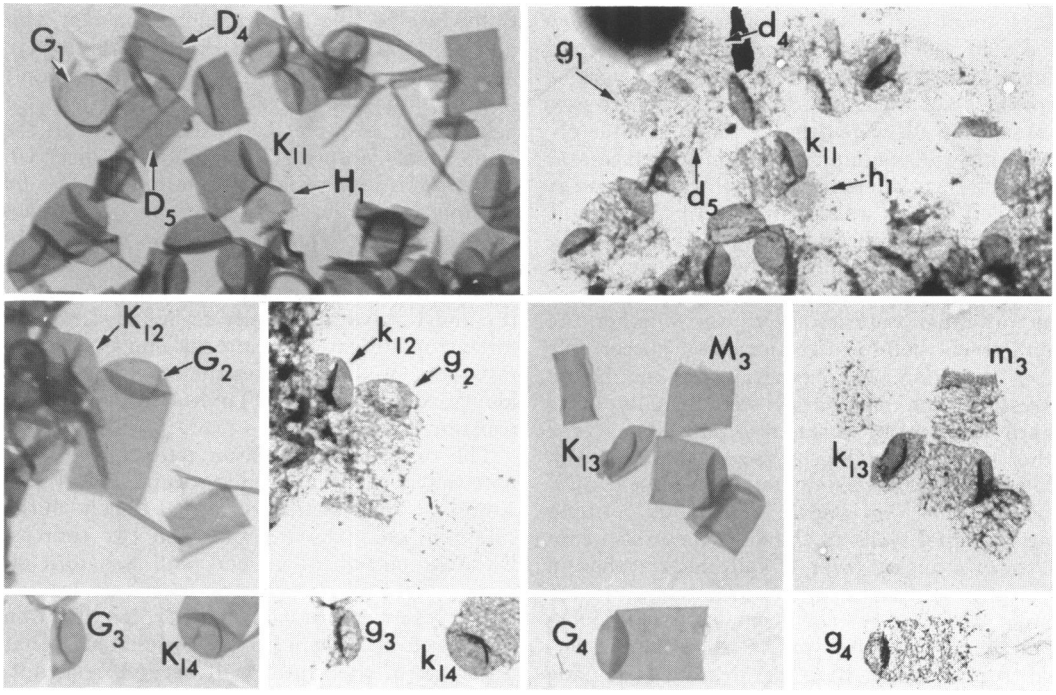

Fig. 5. *B. subtilis autolysin digestion of B. subtilis walls—primarily views of end walls. Notations and procedures as for Fig. 3.* ×7,200.

TABLE 1. *Symbols and frequencies for various types of B. subtilis wall fragments*

Type of fragment	Symbols used in Fig. 3, 4, and 5		No. counted	Frequency (%)
	Before digestion	After digestion		
Partial septal walls-end views				
More digested than side walls	A	a	20	37
As digested as side walls	B	b	32	59
Less digested than side walls	C	c	2	4
Partial septal walls-side views				
More digested than side walls	D	d	20	65
As digested as side walls	E	e	7	23
Less digested than side walls	F	f	4	12
End walls				
Periphery partially resistant to digestion	G	g	71	18
Uniformly partially resistant to digestion	H	h		
Uniformly very resistant to digestion	K	k	319	82
Wall fragments resistant to digestion at end of pieces of side wall	M	m		
Side walls				
Single layer thick	N	n		
Several overlapping layers	P	p		

where the partial septal walls were more resistant, the diameters of the central holes did not change. These results suggest that most end walls were synthesized according to model I (Fig. 2).

This conclusion is supported when data on

digestion of partial septal walls in the side view is considered (Fig. 3, 4, and 5; and Table 1). These examples show the way in which various morphologies were scored. The class where partial septal walls were degraded more completely than side walls (samples D) seems much greater than the class where partial septal walls were as degraded as side walls (samples E). This result is at variance with that found for end-view digestion. However, some of the side view classifications might have been erroneous because the principal criterion used was whether the band seen before digestion was discernible after digestion. In photographs taken before digestion, the boundaries were usually quite sharp where layers of side wall partially covered other such layers (Fig. 3, samples N_1 and P_1). However, after partial degradation these boundaries became very vague. The bands defining partial septal walls in the side views also consisted of a thicker layer of wall material than in the surrounding regions. Therefore, these bands would also have been expected to become indistinct after partial digestion if partial septal walls were as degraded as side walls were. Hence, some of the cases of partial septal walls being more digested than side walls perhaps should be reclassified to join the group where side wall and partial septal walls were equally digested. The other possibility is that in the digestion patterns of partial septal walls in the side views were correctly classified. In this case a possible explanation of the discrepancy in the side view and end-view data might be that in the side view examples most of the partial septal walls probably came from partial septa whose synthesis had just begun. Those partial septal walls near completion would have been relatively rigid disks which could not collapse in the side view to give a single, dark bar. Thus end-view samples of partial septal walls would tend to have more examples from older, partial septa than side-view samples. If the classifications listed in Table 1 are largely correct, then one possibility is that partial septal wall material when first synthesized is more sensitive to degradation than side wall material. In any case all of the partial septal wall degradation studies clearly show that most partial septal walls, unlike the majority of the end walls, are at least as sensitive to digestion as side walls.

If most partial septal walls are not resistant to degradation, then it would be expected that the rings seen in the experiment of Fig. 1 came from end walls by partial degradation. It would therefore be predicted that end walls should have been found which could be digested partially to give ring-like fragments. In fact, this digestion pattern is found (Fig. 5; samples G). The resistant portion of the end wall of sample G_1 (Fig. 5) is not as resistant to digestion as a nearby end (sample K_{11}). Another end in the same field (sample H_1) is uniformly digested, but it too is less resistant than an end close by (sample K_{11}). These observations suggest not only that end walls are modified as predicted in models I and II (Fig. 2) but also that after the modification first covered the entire surface of the end to give a partially resistant structure; further modification made the end even more resistant. Of all structures that could clearly be identified as ends, 18% (Table 1) were not fully resistant to digestion.

The only other digestion pattern that was noteworthy was that seen in samples M (Fig. 3, 4, and 5). The resistant regions at the end of the side wall could have come from two sources. Either a portion of an end wall was torn off during the wall preparation procedure leaving only a resistant fragment left. Or, modification began before the septum was completed so that a portion of the septal wall became resistant. Breaking the cell very close to the septum point would have left a fragment of resisant septal wall attached to the side wall.

DISCUSSION

Our data clearly rule out model III (Fig. 2) as the sole mechanism of end wall synthesis because this model strongly predicts that all septal walls are as resistant to digestion as end walls. Therefore, the important conclusion we can draw is that the end wall resistance was not due to the end wall being made from the beginning from material fundamentally different from side wall material. Rather, the end walls were modified after synthesis. It is possible to imagine that under different growth conditions the modification would proceed at different rates relative to septal wall synthesis so that under some conditions the septal walls might in fact be modified almost as soon as it is made. Then the wall resistance pattern would be as predicted in model III (Fig. 2). In fact, there could even be some heterogeneity within any given culture with different bacteria in the culture modifying septal walls at different stages of septum completion. Under the conditions reported in this paper the most important pathway is that shown in model I because almost all partial septal walls are at least as sensitive as side walls to digestion.

Frehel, Beaufils, and Ryter (4) found that, under appropriate conditions, the wall from one end of a *B. subtilis* bacterium is more resistant

to lysozyme than either the side wall or the wall from the other end. These workers explain their result by suggesting that there was an alteration of end walls after synthesis.

The modification can obviously take one or more of several different forms: addition of new material, removal of old material, or rearrangement of old material. We have no data regarding which one of these changes actually takes place. One possibility is that the teichoic acid is involved in the modification since it is known that the composition of the negatively charged polymer of the wall can affect action by lytic enzymes (6). This possibility can be checked by removing the teichoic acid from walls before digestion with autolysins, and such experiments are in progress.

The function of the modification might be to prevent degradation of the ends. Recent data suggest that *B. subtilis* cell walls grow by uniform intercalation of material along the entire length of the cells (4, 5). Therefore, it is reasonable that side wall should be constantly degraded to permit wall expansion everywhere along the length of the cells. However, in normal growth, the bacteria grow longer without growing wider so there is no obvious need to change the ends once they are made. Thus to protect the ends against attack by the autolysins made by the cells, it is plausible that they should be modified to a resistant form. It is not known whether the septal walls are made of a material different from side walls so that only the former can be modified or if the modification enzymes are only located at the positions of cell ends and partial septa so that modification only occurs in those regions.

The fact that some ends can be found which are not uniformly modified (Fig. 5, samples G) suggests that the modification may not be essential for cell growth. The isolation of a mutant lacking the modification would permit a direct test of this possibility. We are currently investigating procedures for the isolation of such a mutant. A mutant unable to modify the ends would also permit a test of the suggestion from our experiments that new unmodified septal wall material might be even more sensitive than side walls to amidase action.

ACKNOWLEDGMENTS

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